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IN-VITRO PLANT REGENERATION OF SEEDLESS LEMON (*CITRUS LIMON* L. CV. KOKAN LEMON) AND ASSESSMENT OF GENETIC FIDELITY OF REGENERATED PLANT THROUGH DNA PROFILING

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ABSTRACT

The in-vitro plant regeneration protocol was standardized for the multiplication of seedless lemon (Citrus spp. L. cv. Kokan lemon) using juvenile explant. Explants were cultured on full strength Murashige and Skoog (MS Media). The basal medium was supplemented with 16 different concentrations of both 0.8 to 4 mgl-1 BAP and Kinetin respectively. The maximum shoot regeneration in terms of number of shoot and length of shoot was observed when nodal explants were cultured on MS media supplemented with BAP 1 mgl-1 and kinetin 1 mgl-1. It was observed that the time required for establishment of culture was shorter for BAP than kinetin in terms of a number of days. The regenerated plants were transferred for rooting on MS medium with 8 different concentrations of IAA and IBA ranges from 0.5 to 2 mgl-1 respectively. Among this the best results for root induction was observed on IBA 1 mgl-1. For multiplication of shoot, explant was inoculated on media containing BAP and NAA, the optimum results were obtained with a combination of BAP 3 mgl-1 + NAA 0.5 mgl-1.

KEYWORDS: Citrusspp., Multiplication, Regeneration, In-Vitro, Explants & Genetic Fidelity

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Original Article

INTRODUCTION

Citrus has an immensely high consumption rate due to its high nutritive and medicinal values. It also holds high productivity and a large number of fruit products are prepared from it, which leads to its high economic value among the fruit crops throughout the globe. Citrus belongs to the Rutaceae family, is a vitamin C source in human diet. Besides this, it contains volatile oils, limonene, α-pinene, β-pinene, citral, coumarins, bioflavonoids, vitamins, and mucilage (Ashok Kumar *et al*., 2011). Citrus is rich in citric acid and essential oils content which add to its pharmaceutical value. (Bansode *et al.*, 2012). All citrus species have 2n=18 with very similar karyotypic morphology and size. India holds sixth position in citrus productionin the world ranking (NHB, 2015). The origin of citrus is atSouth East Asia mainlytropical and subtropical regions, particularly, India and China. In Maharashtra, *Citrus limon* is commercially cultivated in Akola, Ahmednagar, west Solapur. The varieties being cultivated in Maharashtra are Sai Sharbati and Phule Sharbati developed at MPKV, Rahuri, seedless lemon, Kagzi lime, Vikram, Pramilini, Jai Devi. The variety Sai Sharbati is found to show tolerance against both Tristeza and canker, while Pramilini is only tolerant to canker. The variety Jai Devi is immensely popular for its pleasant aroma.

The propagation of citrus variety is through both sexual and asexual method generally through cutting, layering and budding (Rathore *et al.,* 2006). This put limitation on citrus propagation in the particular season when buds are available. The propagation of commercial varieties of citrus is through the asexual method. In the

case of seedless lemon variety Kokan lemon the method of asexual propagation (air layering) of plants never provide the sufficient planting material therefore to obtain the required quantity and fulfill the market demand of this precious planting material, tissue culture techniques especially micropropagation of citrus, *viz,* somatic embryogenesis, adventitious shoot bud production and axillary enhancement using the nodal segment as explants remains the only viable alternative. However, this method of micropropagation involves a lower risk of somaclonal variation. Hence, the in-vitro propagation despite being so advantageous, it is necessary to establish a suitable protocol to produce genetically stable and identical plants of *Citrus limon* L. cv. Kokan Lemon and testing genetic fidelity of micropropagated plants (Komal *et al.,* 2013). In view of the above, for the commercial application, genetic stable in-vitro regenerated plantlets with large scale multiplication are essential requisite.

MATERIAL AND METHODS

Plant Material and Explant Preparation

Different healthy juvenile explants of 'Kokan Lemon' (seedless) cultivars were excised from mother plants maintained in insect and disease-free condition at Makhmalabad farm of K. K. Wagh College of Agricultural Biotechnology, Nashik (MS). This includes stem segment (without meristematic buds) of size 2.5 to 3 cm, stem segment (with auxiliary bud) size 1.5 to 2 cm and nodal segment (with lateral buds) size 2 to 3 cm. The juvenile explants were sterilized by thoroughly washing with tap water for about 10 to 15 min. The explants were then disinfected using 0.5% Tween-20 liquid soap solution for 10-15 minutes followed by soaking in fungicide (Bavistin) solution (0.1 %) for 20 min and thereafter under laminar airflow the explants were three times washed with sterile distilled water. Streptomycin (100 mg/l) treatment was also given for 10 minutes which was followed by two times sterile distilled water wash. Now, Mercuric chloride ($Hgcl_2$) treatment (0.5%) was given for 5-6 min and again two sterile distilled water wash. For complete sterilization of explants, finally, explants were further treated with 70% ethanol for 30 sec, and then followed by three times wash with sterile distilled water.

Culture Media and Growth Conditions

For *in vitro* culture experiments Murashige and Skoog (1962) basal salt media containing carbon source as 3% sucrose and gelling agent 0.8% agar was prepared. Themedia supplemented with different Auxin and Cytokinin concentrations was used in the experiment with pH maintained at the range of 5.6 to 5.8 before autoclaving for 15 minutes at 121°C and 15 p.s.i. For induction of shoots, the MS media with different concentrations of BAP and Kinetin was used respectively. The cultures were maintained at 25±2 °C with 16/8 h (light/dark) photoperiod with the help of cool white fluorescent tubes (Philips, India) in a growth room. After 30 -35 days interval, explants were subcultured. Finally, the regenerated shoots were transferred further to the shoot multiplication media followed by root induction media. Data obtained from cultures including days required for induction of shoot, number and length (cm) of shoots present per explant, after45 days of culture establishment and Shoot induction frequency was calculated.

Root Induction

Multiplied shoots were then transferred for rooting on ½ MS supplemented with four different concentrations of growth hormone Auxin, IAA and IBA respectively. Different treatments were tried out to find out which combination induces higher rooting. After the inoculation of *in-vitro* grown shoots, rooting was observed for about 15 days. Data obtained on root induction frequency, days required for root induction, developed number and length (cm) of roots present per explant,

after 45 days of culture establishment. The root induction frequency was also calculated. Then the plantlets were prepared for hardening.

Hardening of Explant

The healthy regenerated plantlets were obtained after rooting of the shoots supplemented with the ½ MS and Auxin. These plantlets were thoroughly washed with tap water without causing any damage to the roots to remove agar particles. Further treatment of 0.05% bavistin was given to the rooted plantlets then finally the *in-vitro* generated plantlets were potted in a mixture comprising of vermicompost, coco peat and garden soil pH 5.4 mixed in a combination of 1:1:1 in hardening glasses for primary hardening. Then the plantlets were covered with a transparent plastic bag and transferred to the greenhouse of the college farm at 28˚C. After seven days interval gradually remove the cover, initially for 3 h followed by 6 h and 12 h during day and then for the next three days during night put-off lights keep plants uncovered. Then gradually increased the period of keeping the plantlets uncovered and after 15 days plantlets were kept in shade, outside the room. Finally, for next 10 days the Sun exposure was given for their acclimatization to the natural environment. Meanwhile, the nutrient medium was also removed gently. And finally, for secondary hardening, the plantlets were transferred to the greenhouse of college farms in the plastic pots.

Experimental Design and Statistical Analysis

The experiment was designed as per the RCBD (Randomized Complete Block Design) with three replications per treatment. Statistical analysis was carried out by performing ANOVA to determine the significant difference between the treatment means. Further by using the software SPSS ver.16.0 the comparison between the treatment mean was made using DMRT (Duncan's Multiple Range Test).

Genetic Fidelity Analysis by RAPD

Genetic assessment of regenerated plant was done by polymerase chain reaction (PCR) based markers. Firstly, genomic DNA was isolated done by CTAB method from juvenile leaves sample collected from mother plant and *in vitro* regenerated plantlets respectively. The quantification of isolated DNA was done by UV-vis spectrophotometer and its quality check was done using 0.8% agarose gel electrophoresis. For RAPD assay twelve different primers were used. For PCR reaction mixture 25 µl was prepared. It contains 1 µl of Template DNA (~100 ng), 2.5 µl 10X PCR buffer, 2.0 µl dNTPs (10 mM), 1.5 µl $MgCl_2$ (1.5 mM), 1 µl Primers (10 mM), 0.5 µl *Taq* Polymerase (5 U) and 16.5 µl sterile distilled water. Finally, DNA amplification was done in a DNA thermal cycler (Eppendorf, USA). The thermal profile used for amplification consists of one cycle for initial denaturation at 95^0C for 5 min, followed by final denaturation at 94^0C, 1 min with 40 cycles, 1 min. at 37°C annealing temperature, 1 min. at 72^0C (polymerization), with a final extension of 10 min at 72^0C for a final extension. The amplicons were resolved on 1.2 % agarose gel through electrophoresis and visualized in a gel documentation unit (UVITEC). The amplicon size was determined by the 100 bp molecular size marker (Gene Ruler).

RESULTS

Effect of BAP and Kinetin on Proliferation of Shoot

The MS medium supplemented with various concentrations of 0.8-4 mgl^{-1} BAP and 0.8-4 mgl^{-1} Kinetin shows the different morphogenic response of the explant presented in Table 1 and 2 respectively. The highest shoot induction frequency (93.10%) of explant was observed in the explant inoculated on the medium supplemented with 1 mgl^{-1} BAP whereas in the

case of 1 mgl^{-1} kinetin the shoot induction frequency observed was (60%). The lowest frequency (16.66%) observed on kinetin 3 mgl^{-1}. It was observed that the explant inoculated on the lower level of cytokinin took less time than the explants inoculated at a higher level to bud break. The number and length of shoot explant vary with the varying concentration of BAP and Kinetin. The maximum (2.8) number of shoot per explant and shoot length (3.2 cm) was recorded in the MS medium supplemented with BAP 1 mgl^{-1} while in case of 1 mgl^{-1} Kinetin maximum (1.8) number of shoot per explant and shoot length (3.2 cm) was observed. But, as the concentration increases the number of shoot declines also the length of shoots on 4 mgl^{-1} of BAP was shorter (1.3 cm).

Table 1: Effect of Growth Hormones (BAP) on Shoot Induction

Treatment(T)	MS + BAP (mg/L)	No. Shoots Per Explant	Length of shoot(cm)	Shooting Induction Frequency (%)	Days to Shoot Induction
T1	0.8	2.3±0.33	2.8±0.26	76.50	12±0.53
T2	**1.0**	**2.8±0.30**	**3.2±0.19**	**93.10**	**12±0.37**
T3	1.4	2.6±0.31	2.5±0.17	86.40	18±0.41
T4	1.5	2.3±0.33	2.2±0.20	76.56	17±0.34
T5	1.8	2.3±0.39	2.3±0.14	76.56	22±0.57
T6	2.0	1.6±0.34	1.8±0.04	53.50	25±0.32
T7	3.0	1.3±0.31	1.6±0.15	43.33	25±0.53
T8	4.0	01±0.29	1.3±0.08	33.25	26±0.60

Table 2: Effect of Growth Hormones (Kinetin) on Shoot Induction

Treatment (T)	MS + Kinetin (mg/L)	No. Shoots Per Explant	Length of shoot (cm)	Shooting Induction Frequency (%)	Days to Shoot Induction
T1	0.8	1.6±0.32	2.5±0.12	53.33	23±0.58
T2	**1.0**	**1.8±0.57**	**3.2±0.08**	**60.00**	**20±0.60**
T3	1.4	1.3±0.33	2.5±0.03	43.33	23±0.57
T4	1.5	1.5±0.31	2.2±0.05	50.21	26±0.72
T5	1.8	1.2±0.36	2.4±0.12	40.10	27±0.66
T6	2.0	0.8±0.32	1.8±0.23	26.66	27±0.54
T7	3.0	0.5±0.03	1.3±0.03	16.66	28±0.57
T8	4.0	0.5±0.03	1.4±0.05	16.67	28±0.66

Effect of BAP and NAA on Shoot Proliferation

The effect of the combination of auxin and cytokinin (NAA+BAP) was also observed on bud breaking frequency and days required to obtain it, number and length of the regenerated shoot (Table 3) The combination of BAP 3 mgl^{-1} and NAA 0.5 mgl^{-1} shows maximum shoot proliferation (2.8) and highest shoot length (3.7 cm). While the BAP 0.8 mgL^{-1} and 0.5 mgl^{-1} NAA show minimum shoot proliferation (1.0) and lowest shoot length (1.8 cm). Early induction of shoot was observed on 12 days while shoot induction was delayed to 26 days due to the media containing increased BAP concentration. BAP in combination with NAA with increasing concentration promotes shoot proliferation and shoot length. While when the BAP alone was used in the media it shows a decrement in length of shoot and shoot proliferation.

Table 3: Effect of Hormones (BAP and NAA) on Shoot Multiplication after 4 Weeks

Treatment (T)	MS + Hormone (mg/L)		Length of Shoot (cm)	Number of Shoot
	BAP	NAA		
T1	0.8	0.5	1.8±0.08	1.0±0.05
T2	1.0	0.5	2.0±0.08	1.3±0.33

T3	1.2	0.5	2.1±0.15	2.3±0.30
T4	1.4	0.5	1.9±0.07	1.6±0.36
T5	1.5	0.5	2.2±0.06	1.6±0.31
T6	2.0	0.5	2.4±0.03	2.0±0.57
T7	**3.0**	**0.5**	**2.8±0.05**	**2.6±0.33**
T8	4.0	0.5	2.7±0.08	2.3±0.36

Root Induction

For root induction the regenerated shoots were transferred on ½ MS media supplemented with 4 different concentrations of IAA and IBA (0.5 to 2 mgl^{-1}) respectively. After 12 to 15 days root induction occurred. The present study has led to an observation that both the media, ½ MS + IAA and ½ MS + IBA respectively shows almost equal *in vitro* root induction but the optimum and early root induction was obtained at different concentrationat1.5 $mgl^{-1}$for IAA while for IBA at 1 mgl^{-1}

Table 4: Effect of Growth Hormones (IAA) on Root Induction

Treatment	MS + Hormone (mg/L)	No. Roots Per Explant	Length of Root(cm)	Rooting Induction Frequency (%)	Days to Root Induction
(T)	IAA				
T1	0.5	1.0±0.05	1.0±0.05	33.33	26±0.05
T2	1.0	1.8±0.64	1.2±0.10	60.12	28±0.57
T3	**1.5**	**2.0±0.57**	**3.0±0.46**	**66.66**	**23±0.82**
T4	2.0	1.5±0.33	2.2±0.06	50.00	26±0.33

Table 5: Effect of Growth Hormones (IBA) on Root Induction

Treatment	MS + Hormone (mg/L)	No. roots Per Explant	Length of Root(cm)	Rooting Induction Frequency (%)	Days to Root Induction
(T)	IBA				
T1	0.5	1.3±0.33	2.5±0.05	43.33	24±0.83
T2	**1.0**	**2.0±0.05**	**3.1±0.05**	**66.66**	**22±0.33**
T3	1.5	1.6±0.32	1.4±0.05	53.33	24±0.66
T4	2.0	1.3±0.35	1.2±0.03	43.33	23±0.66

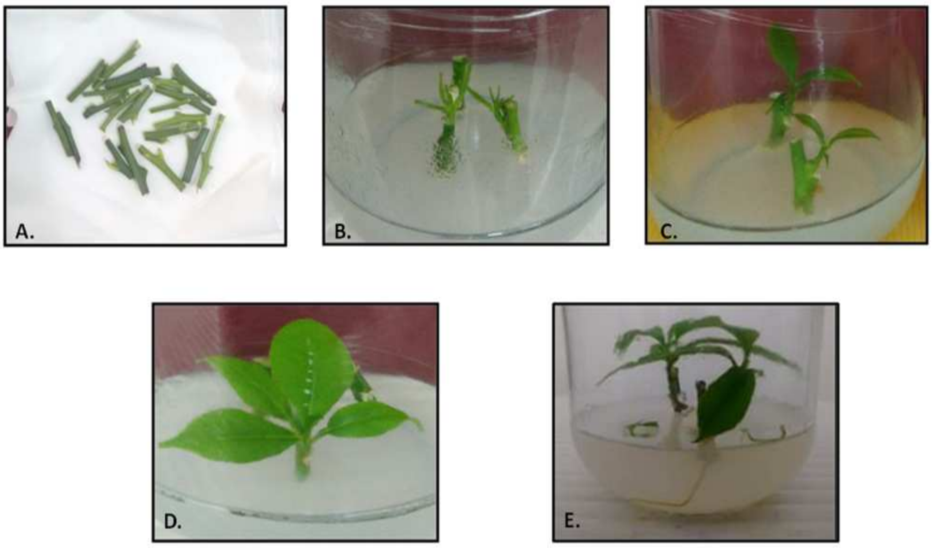

Plate 1: A) Sterilize Nodal Explant from Mother plant B) Establishment of Shoots from explants C) Shoot Initiation D) Shoot Proliferation E) Root induction

Genetic Fidelity Assessment of Regenerated Plantlets

For the genetic uniformity assessment of *in vitro* regenerated plants, few regenerated plants of *Citrus limon* were taken and analyzed for RAPD analysis. In the present study, out of the eight Primers were screened, Primer-4 and Primer-7 show the maximum amplification while the Primer-3, Primer-11 and Primer-17 show lesser amplification. Primer-1 did not show any amplification. The maximum numbers of amplified fragment generated by Primer-7 were 8 bands followed by Primer-4 with 6 bands. The amplified products generated by Primer 3 and with Primer-4 are presented in Plate 2. The results obtained from the 20 amplified products were all monomorphic bands and show uniformity with the mother plant**.** From the present investigation, it could be concluded that, *in vitro* regenerated plants possess genetic uniformity with their parental type hence are suitable for commercialization purposes. In the case of Citrus species, it has previously been reported that for the confirmation of the genetic uniformity of the micro propagated plants RAPD analysis has been made(Khawale and Singh, 2005). Plant tissue culture has proved to be a potential tool by producing disease-free health plants within a short period of time on large-scale and even in the offseason, with a high fidelity index.

Table 6: Primer Sequence used for Genetic Fidelity

Sr. No	RAPD Primers	Sequence (5' → 3')	Total Number of Amplified Products	Presence of Novel Bands
1	Primer-1	ACACTTCTGC	-	
2	Primer-3	TCGGCCATAG	2	-
3	Primer-4	CCCAGTCACT	6	-
4	Primer-7	GACCGCTTGT	8	-
5	Primer- 11	GGATGAGACC	3	-
6	Primer- 15	AGGTGACCGT	5	-
7	Primer-17	GAACCTGCGG	2	-
8	Primer-19	CAGCTCACGA	4	-
	Total amplified fragments		20	

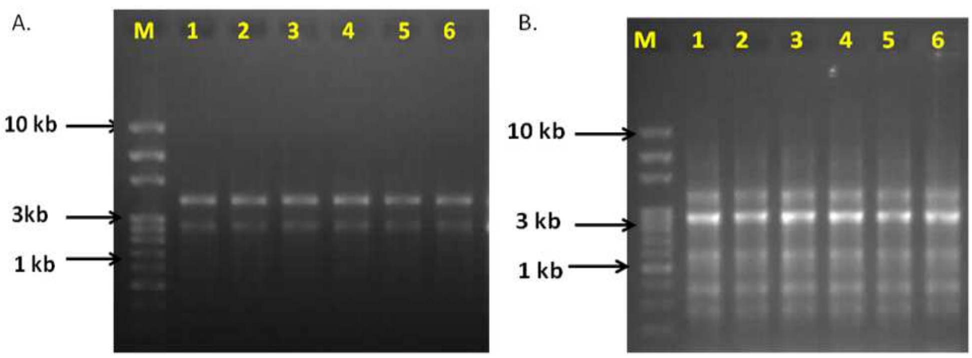

Plate 2: A)DNA Amplification Profile by RAPD Marker (Primer-3) (Lane M- Ladder, lane 1- Mother Plant, Lane 2 to 6 – Regenerated Plants). B) DNA Amplification Profile by RAPD Marker (Primer-4) (lane M- Ladder, Lane 1- Mother Plant, lane 2 to 6– Regenerated Plants).

DISCUSSIONS & CONCLUSIONS

Citrus diseases are mostly transmitted via seed so *in vitro* regeneration of seedless lemon will also help to regenerate disease-free plants. The present work was done for the standardization of *in vitro* plant regeneration protocol of *Citrus limon* L cv. Kokan lemon (Seedless lemon) as there have been only a few reports of the work done on the seedless lemon. In this study, various concentrations and combinations of plant growth regulators were tried for shoot proliferation, multiplication, and root induction. The maximum results were obtained on the media supplemented with 1 mgl^{-1} BAP for

shoot proliferation while BAP 3 mgl^{-1} +NAA 0.5 mgl^{-1} was maximum for shoot multiplication (Table 3). Similarly, the maximum multiple shoots in nodal explants were reported by Shawk at Ali and Bushra Mirza (2006) obtained bud breaking with BAP. Increasing the concentration of BAP in combination with NAA has a promotory effect on shoot proliferation and shoot length. While when the BAP alone was used in the media show a decrement in the length of shoot and shoot proliferation. It has been reported that for optimal multiple shoot proliferation in mature trees explant, the nutritional requirement vary with lemon age, genotypes and physiological conditions. The endogenous levels of phytohormones of the donor tissue and the influence of the plant growth regulators (PGRs) added exogenously in the culture medium results in the morphogenic development. response of explants cultured *in-vitro*. For the shoot regeneration, cytokinin is effective when used alone or in combination with an auxin. It has been recorded from the present study that, BAP has superiority over Kinetin. Cytokininhas major role in shoot induction by activation of meristems which ultimtely results in shoot proliferation (Murashige and Skoog, 1962). The major problem in citrus plant has been reported as the low efficiency of rooting. Several reports on rooting with different concentration of auxin in the medium are known. IAA or IBA is the auxin which is commonly used for rooting induction of in-vitro regenerated shoots of citrus (Cheong, E. *et al.*, 2003). The IBA 1mg/l has been reported as the best rooting auxin (Table 5) however, in some other reports IAA is used to induce rooting. Herewith IAA the highest rooting was observed at 1.5mgl^{-1}. Similarly, rooting in regenerated shoots from nodal explant of Citrus reticulate Lime was obtained in half-strength MS medium supplemented with IBA by Al-khayri and Al- Bahrany (2001).

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